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Novel genes involved in pathophysiology of gonadotropin-dependent adrenal tumors in mice



Milena Doroszko^a, Marcin Chrusciel^a, Kirstine Belling^b, Susanna Vuorenoja^a,
Marlene Dalgaard^b, Henrik Leffers^b, H. Bjørn Nielsen^b, Ilpo Huhtaniemi^{a,c},
Jorma Toppari^{a,d}, Nafis A. Rahman^{a,e,*}

^a Department of Physiology, Institute of Biomedicine, University of Turku, Finland

^b DTU Multi-Assay Core, Department of Biotechnology and Biomedicine, Technical University of Denmark, Kongens Lyngby, Denmark

^c Institute of Reproductive and Developmental Biology, Imperial College London, London, UK

^d Department of Pediatrics, Turku University Hospital, Turku, Finland

^e Department of Reproduction and Gynecological Endocrinology, Medical University of Białystok, Poland

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ABSTRACT

Specific inbred strains and transgenic inhibin- α Simian Virus 40 T antigen (inh α /Tag) mice are genetically susceptible to gonadectomy-induced adrenocortical neoplasias. We identified altered gene expression in prepubertally gonadectomized (GDX) inh α /Tag and wild-type (WT) mice. Besides earlier reported *Gata4* and *Lhcgr*, we found up-regulated *Esr1*, *Prlr-rs1*, and down-regulated *Grb10*, *Mmp24*, *Sgca*, *Rerg*, *Gnas*, *Nfatc2*, *Gnrhr*, *Igf2* in inh α /Tag adrenal tumors. Sex-steroidogenic enzyme genes expression (*Srd5a1*, *Cyp19a1*) was up-regulated in tumors, but adrenal-specific steroidogenic enzyme (*Cyp21a1*, *Cyp11b1*, *Cyp11b2*) down-regulated. We localized novel *Lhcgr* transcripts in adrenal cortex parenchyma and in non-steroidogenic A cells, in GDX WT and in intact WT mice. We identified up-regulated *Esr1* as a potential novel biomarker of gonadectomy-induced adrenocortical tumors in inh α /Tag mice presenting with an inverted adrenal-to-gonadal steroidogenic gene expression profile. A putative normal adrenal remodeling or tumor suppressor role of the down-regulated genes (e.g. *Grb10*, *Rerg*, *Gnas*, and *Nfatc2*) in the tumors remains to be addressed.

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1. Introduction

The adrenal cortex is the steroidogenically active part of the adrenal gland consisting of 3 layers, i.e., zona glomerulosa, zona fasciculata and zona reticularis in humans, or x-zone in mice (corresponding to human zona reticularis), which is a vestige of the fetal adrenal cortex (Pihlajoki et al., 2015). Each layer expresses specific steroidogenic enzymes that results in the zone-specific production of mineralocorticoids, glucocorticoids or androgens, respectively (Pihlajoki et al., 2015). In contrast to human zona reticularis, the x-zone in postnatal mice does not produce androgens due to inactivation by methylation of *Cyp17a1*, the enzyme directing steroidogenesis to production of sex steroids (Pihlajoki et al., 2015). The highly differentiated cells of the adrenal zones

do not proliferate, but are replaced by new cells migrating towards medulla from the subcapsular niche of stem/progenitor cells (Pihlajoki et al., 2015). This pool of progenitors has a common origin with gonadal somatic cells, which forms the adreno-gonadal primordium in early embryonic life (Laufer et al., 2012; Pihlajoki et al., 2015). Interestingly, following gonadectomy, some strains of inbred mice (DBA/2J, NU/J) develop adrenocortical neoplasms that structurally and functionally resemble ovarian theca and granulosa cells (Bielinska et al., 2005; Bielinska et al., 2003).

Prepubertally gonadectomized inbred DBA/2J or NU/J mice develop adrenal adenomas with two types of neoplastic cells, non-steroidogenic A cells (gonadectomy-independent) originating from the subcapsular region (Hofmann et al., 1960) and larger lipid-laden B cells (gonadectomy-dependent) located between the foci of A cells (Rosner et al., 1966), but still with unclear clonal origin. Only the B cells are steroidogenic and express gonad-specific genes such as *Lhcgr*, *Gata4*, and the steroidogenic enzymes *Cyp17a1* and *Cyp19a1*, resulting in a significant estrogen production (Bielinska

* Corresponding author. University of Turku, Institute of Biomedicine, Department Physiology, Kiinamyllynkatu 10, Turku 20520, Finland.

E-mail address: nafis.rahman@utu.fi (N.A. Rahman).

et al., 2003; Chrusciel et al., 2013; Krachulec et al., 2012). However, unlike the aforementioned mouse models, human adrenocortical tumors (ACT) produce mostly adrenal steroids (Stojadinovic et al., 2003) and sex steroid production occurs only in 5% of all ACT (Moreno et al., 2006). Interestingly, a large group of ACTs respond to luteinizing hormone (LH)/choriogonadotropin (CG) stimuli (Carlson, 2007) with production of cortisol, aldosterone or androgens. This situation is usually correlated with chronically elevated LH (menopause) or hCG (pregnancy) levels (Alevizaki et al., 2006; Carlson, 2007; Saner-Amigh et al., 2006).

A common cause for human adrenal malignancies are TP53 germline (80% of pediatric and 6% of adult ACT cases) and somatic (25–70% of adult ACC) mutations (Lerario et al., 2014). Inactivation of p53 promotes development of neoplastic cells by enabling progression through the S-phase of the cell cycle, despite DNA damage (Levine et al., 1991). Modeling of p53 inhibition is possible by transgenic expression of the Simian Virus 40 T-antigen (SV40Tag), and to date it has been successfully used to induce malignancies in various mouse organs (Hudson and Colvin, 2016). Identifying the molecular mechanisms leading to tumor formation and progression in animal models expressing SV40Tag might therefore provide novel insights into human tumors induced by p53 inactivation.

Inh α /Tag mice express SV40Tag oncogene under the inhibin α promoter in a C57Bl/6 genetic background and have been well established as a model for gonadal and adrenocortical tumorigenesis (Kananen et al., 1996; Rahman et al., 2004; Rilianawati et al., 1998). Intact inh α /Tag mice have been extensively used to study gonadal tumorigenesis (Kananen et al., 1996; Kiiveri et al., 1999; Rahman et al., 2004; Vuorenoja et al., 2007). To induce adrenocortical tumorigenesis, the inh α /Tag mice need to be prepubertally gonadectomized (Kananen et al., 1996). Generally, females develop adrenal tumors faster and to larger size than males, with high individual variations of tumor volume and histological appearance (Rahman et al., 2004). The tumors express highly inhibin alpha (INHA), transcription factor GATA4 and luteinizing hormone/chorionic gonadotropin receptor (LHCGR) (Kananen et al., 1996; Kiiveri et al., 1999; Rahman et al., 2004; Rilianawati et al., 1998). Adrenocortical tumorigenesis in inh α /Tag model has been shown to be strictly gonadotropin dependent. Crossbreeding with mice lacking gonadotropin releasing hormone (GnRH) (*hpg* mice) or treatment with the GnRH antagonist cetrorelix prevented tumor formation (Rilianawati et al., 2000). Moreover, crossbreeding the inh α /Tag mice with transgenic mice expressing bovine *Lhb* fused with the human chorionic gonadotropin β -subunit C-terminal peptide (bLH β -CTP) (Risma et al., 1995), resulted in a 10-fold higher LH levels, and enhanced simultaneous development of gonadal and adrenocortical tumors (Mikola et al., 2003). Until now, molecular mechanisms of the adrenocortical tumor formation and progression in conjunction with the elevated LH and subsequent LHCGR up-regulation remain largely unknown.

In this study, we used the inh α /Tag male mouse model to analyze the molecular basis of the LH-dependent adrenal tumorigenesis. In doing so, we identified and validated novel biomarker genes, complementing the earlier established LHCGR and GATA4 markers. We also revisited the *Lhcgr* transcript localization in normal and neoplastic adrenal cells.

2. Materials and methods

2.1. Experimental animals and tissue preparation

Male inh α /Tag and negative control littermate (C57Bl/6N) mice were used as the animal model. Mice were kept in a specific pathogen-free surrounding with controlled light (12 h light, 12 h darkness) and temperature (21 \pm 1 $^{\circ}$ C), fed with mouse chow SDS

RM-3 (Witham, Essex, UK) and tap water *ad libitum*. The Ethics Committees for animal experimentation of the Turku University and the State Provincial Office of Southern Finland approved the animal experiments.

Prepubertal gonadectomy in inh α /Tag and WT mice was performed at 21–24 days of age. Surgery was performed under isoflurane (2–4%) anesthesia (Isoflo, Orion Pharma, Turku, Finland) and Temgesic (buprenorphine, 0.1 mg/kg/8 h) (Schering-Plough, Brussels, Belgium) was administered as mid- and post-operative analgesia. Seven-month-old GDX WT and inh α /Tag, and intact WT mice were sacrificed by exsanguination under isoflurane anesthesia. Tissue weights were recorded, adrenals snap-frozen in liquid nitrogen, and/or fixed with 4% paraformaldehyde (PFA). From each mouse about 900 μ l – 1 ml of blood was collected into a tube consisting 100 μ l of 0.5 M sterile EDTA solution. Plasma was fractioned by centrifugation at 3000 RPM for 10 min in 4 $^{\circ}$ C and stored in –80 $^{\circ}$ C for further analysis.

2.2. Hormone measurements

LH levels in plasma were measured by immunofluorometric assay (DELFI; PerkinElmer) as described previously (Haavisto et al., 1993). Progesterone and testosterone blood plasma concentrations were analyzed by Elecsys[®] Progesterone II and Testosterone II assays (Roche Diagnostics, Basel, Switzerland), using Cobas e411 immunoanalyzer (Roche Diagnostics, Basel, Switzerland). Detection limits for progesterone and testosterone were 0.10 nmol/l and 0.09 nmol/l respectively.

2.3. Microarray

To identify novel biomarker genes for adrenocortical tumors, we compared adrenals of 7mo GDX inh α /Tag and WT males. Total RNA was extracted from frozen tissues ($n = 8$ /group) using RNeasy Mini Kit (Qiagen, Germantown, MD), re-suspended in 50 μ l of nuclease-free water (Promega, Madison, WI), quantified spectrophotometrically (NanoDrop; Thermo Fisher Scientific, Waltham, MA) and then qualified using Bioanalyzer nano kit (Agilent Technologies, Santa Clara, CA). The RNA stock was divided into two batches in order to use the same template for both microarray and gene expression validation by qPCR. RNA was transcribed ($n = 4$ /group) using the MessageAmp II aRNA Amplification Kit (Thermo Fisher Scientific) and applied to Agilent whole mouse genome oligo microarrays 4 \times 44K (#GPL7202, Agilent Technologies) accordingly to manufacturer's protocol (Agilent Technologies). The readout was loaded into the limma R/Bioconductor package, normalized between arrays using the quantile normalization. After performing a row-wise *t*-test, fold changes were log2-transformed. Genes with fold change higher than 1.5 fold and *p*-value lower than 0.05 were considered as differentially expressed. Heatmaps were generated using the gplot package in R. The list of significantly differentially expressed genes were uploaded to GOrilla (Eden et al., 2009) and separate, process-based enrichment lists for up- and down-regulated genes were generated.

2.4. Real-time quantitative PCR (qPCR)

Prior to qPCR, 900 ng of total RNA was DNaseI treated (Thermo Fisher Scientific) and transcribed (60 min in 37 $^{\circ}$ C) using DyNAmo[™] cDNA Synthesis Kit (#F470, Thermo Fisher Scientific). qPCR was carried out on a CFX96 Real Time PCR Detection System (BioRad, Vienna, Austria), using the DyNAmo[™] Flash SYBR[®] Green qPCR Kit (#F415, Thermo Fisher Scientific) with 15 ng of cDNA template in total reaction volume of 20 μ l, in duplicates. Conditions were as follows: 95 $^{\circ}$ C for 7 min, [95 $^{\circ}$ C for 15 s, 54–62 $^{\circ}$ C for 15 s,

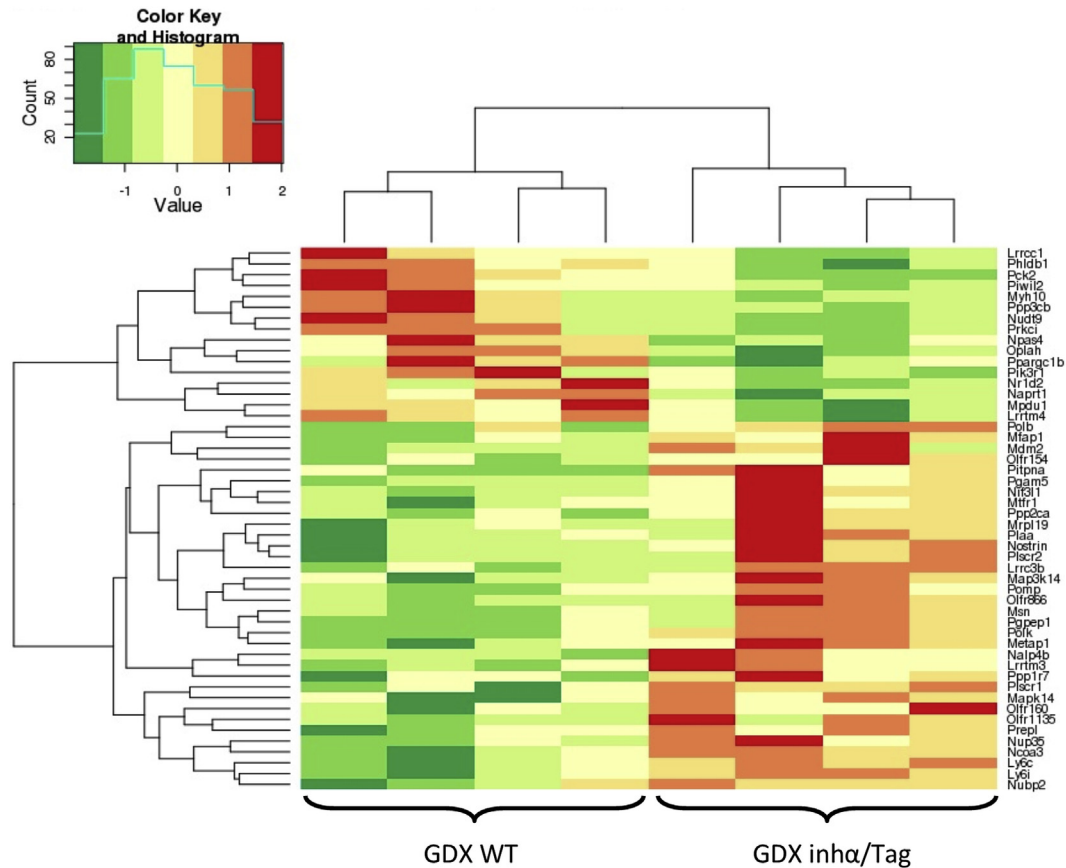


Fig. 1. Heatmap of the 50 most differentially expressed genes in GDX inh α /Tag vs. GDX WT male mice. In the microarray we found 1810 up-regulated and 1606 down-regulated genes in tumorous (GDX inh α /Tag) vs. healthy (GDX WT) male mice adrenal glands. Green represents up-regulated and red stands for down-regulated probes in GDX inh α /Tag vs. GDX WT (n = 4/group).

72 °C for 15s] x 40, 72 °C for 3 min, 65–95 °C melt curve. Sequences of primers are listed in Suppl. Table S1. Ct for each gene of interest was normalized by 2–3 reference genes: cyclophilin A (*Ppia*), β -glucuronidase (*Gusb*), and hypoxanthine phosphoribosyltransferase (*Hprt1*), validated for each experiment using Bio-Rad CFX Manager software (BioRad). Gene expression was calculated using qBase MSeExcel VBA applet (Helleman et al., 2007).

2.5. Immunohistochemistry

PFA fixed paraffin embedded adrenal glands from intact and GDX WT, and inh α /Tag (n = 4/group) were sectioned $\pm 5 \mu\text{m}$ and stored in darkness at +4 °C. Antigens were retrieved in 10 μM citrate buffer (pH6), washed in TBS with 0.1% Tween20 (#P1379, Sigma-Aldrich, Saint Louis, US-MO). Primary and secondary antibodies were listed in Suppl. Table S2. HRP signal was visualized after 10 min incubation with Liquid DAB + Substrate Chromogen System (Dako, Glostrup, Denmark). Slides were scanned by Panoramic 250 Slide Scanner (3DHISTECH Ltd., Budapest, Hungary).

2.6. Densitometric analysis

Images from good-quality representative areas (at least 10 from each slide) of GDX inh α /Tag and WT adrenals (n = 4/group) were acquired with Panoramic Viewer (3DHISTECH Ltd.) at 60X magnification and analyzed by Fiji (Schindelin et al., 2012). In brief, Hematoxylin-Diaminobenzidine (H-DAB) color deconvolution was performed and units of intensity occupied by DAB staining (grey)

were counted. Intensity numbers were transformed into optical density (OD) values using formula $\text{OD} = \log(255/\text{Mean intensity})$.

2.7. In situ hybridization

We used RNAscope[®] 2.5 HD Reagent Kit-BROWN (#322300, Advanced Cell Diagnostics) for *in situ* hybridization (ISH) (Wang et al., 2012) with predesigned probes for *Lhcgr*, *Ppib* (control reference probe) and *DapB* (from *Bacillus S.*, nonsense probe) (n = 4/group). Hybridization was performed accordingly to manufacturer's protocol. Slides were scanned by Panoramic Midi FL slide scanner (3DHISTECH Ltd.).

2.8. Statistical analysis

Numerical data were shown as mean \pm SEM. Graphs and statistical analysis using *t*-test (for 2 experimental groups) or one-way ANOVA (for groups bigger than 2) followed by the Dunnett's comparison test were performed using Graph Pad Prism 6 (Graph Pad Software, San Diego California, USA). We considered *p* values < 0.05 statistically significant.

3. Results

3.1. Gene expression profiling of adrenal tumors of the inh α /Tag mice

cDNA microarray analysis from GDX inh α /Tag males and their

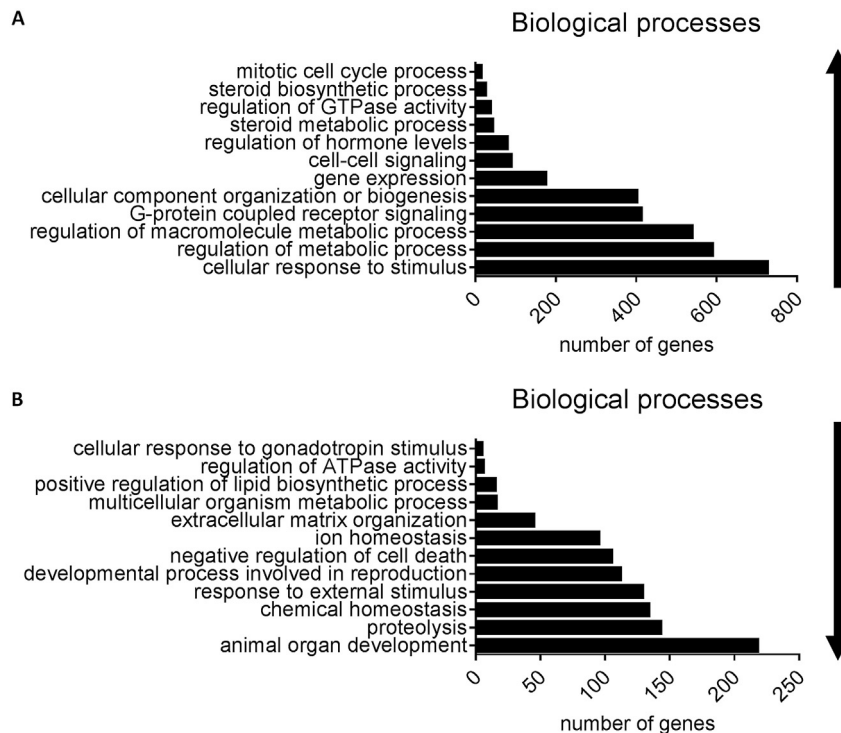


Fig. 2. Gene Ontology (GO) classification (biological processes) of dysregulated genes in adrenocortical tumors vs. GDX WT male mice adrenals. Significantly dysregulated genes were classified based on the biological processes they are involved in. The analysis shows the number of genes up- (Fig. 2A) or down-regulated (Fig. 2B) in the adrenocortical tumors of GDX *inh α /Tag* vs. GDX WT.

control GDX WT (C57Bl/6N) male littermates revealed 1810 up-regulated and 1606 down-regulated genes. A heatmap of unsupervised hierarchical clustering of 50 genes is presented in Fig. 1. Full results of the cDNA microarray analysis are available through ArrayExpress (accession number E-MTAB-5310). Gene Ontology (GO) enrichment analyses (Eden et al., 2009) was performed separately for significantly up- (Fig. 2A) and down- (Fig. 2B) regulated genes to classify them into biological processes. Among up-regulated biological processes were cellular response to stimulus, G-protein coupled receptor (GPCR) signaling, cellular component organization or biogenesis, gene expression, cell-cell signaling, regulation of hormone levels, steroid biosynthetic and metabolic process, and mitotic cell cycle processes. The most prominent down-regulated biological processes were animal organ development, proteolysis, negative regulation of cell death, ion homeostasis, extracellular matrix organization and cellular response to gonadotropin stimulus.

3.2. Validation and localization of potential biomarkers

The validation of selected aberrantly expressed genes by qPCR confirmed significant up-regulation of estrogen receptor alpha (*Esr1*) and long isoform of prolactin receptor (*Prlr-rs1*) (Fig. 3A), whereas *Esr2* was not significantly changed vs. WT control (Fig. S1A). Within down-regulated novel genes, there were extracellular matrix regulating compounds such as sarcoglycan delta (*Sgcd*) and matrix metalloproteinase 24 (membrane-inserted) (*Mmp24*). Other down-regulated genes were cell growth related genes including insulin-like growth factor 2 (*Igf2*), E3 ubiquitin-protein ligase NEDD4 (*Nedd4*), growth factor receptor-bound protein 10 (*Grb10*), RAS-like, estrogen-regulated, growth inhibitor (*Rerg*); gonadotropin-releasing hormone receptor (*Gnrhr*). Moreover, we identified down-regulation of G-protein coupled receptor signaling downstream mediators, guanine nucleotide binding

protein, alpha stimulating complex locus (*Gnas*) and nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 (*Nfatc2*) (Fig. 3A). The gene expression profile in males showed identical pattern with that in females (Fig. S1B). Therefore further validation studies were conducted only in males. ESR1 protein expression was found in cells of zona glomerulosa of the normal adrenal, whereas almost all tumor cells expressed ESR1 abundantly (Fig. 3B and C). Protein expression of GRB10 (Fig. 3D–E), RERG (Fig. 4A–B), GNAS (Fig. 4C–D), NFATC2 (Fig. 4E–F) were abundant in zona glomerulosa of the normal adrenal, whereas faint or absent in the tumor foci of *inh α /Tag* adrenals. Moreover, gonadectomy had no impact on cellular localization of the analyzed biomarkers in WT adrenals (Fig. S1C–G).

3.3. *Lhcgr*, *GATA4* and *GATA6* expression and localization in GDX normal and neoplastic adrenals

Expression of *Lhcgr* (Fig. 5A) and *Gata4* (Fig. 5B) was up-regulated, whereas *Gata6* (Fig. 5C) was down-regulated in the tumorigenic adrenals vs. GDX WT. Using RNAscope *in situ* hybridization (Wang et al., 2012), we revisited the localization of *Lhcgr* expression. GATA4 and GATA6 immunohistochemical localization was used as validated markers of neoplastic/normal adrenocortical cells (Chrusciel et al., 2013; Rahman et al., 2001). *Lhcgr* transcripts were found in foci scattered in GDX WT adrenal cortex (Fig. 5D), in most of A cells in GDX WT (Fig. 5E) and abundantly in the adrenocortical tumors of *inh α /Tag* mice (Fig. 5F). Moreover, in intact WT mice *Lhcgr* localization was similar to GDX WT (Fig. S1H–I). GATA4 was not expressed in the GDX WT adrenal glands (Fig. 5G), but was abundant in most of the A (Fig. 5H) and adrenocortical tumor cells (Fig. 5I). GATA6 staining was abundant in the GDX WT adrenal gland (Fig. 5J), absent in A cells (Fig. 5K) and displayed only a weak signals in the tumor foci (Fig. 5L).

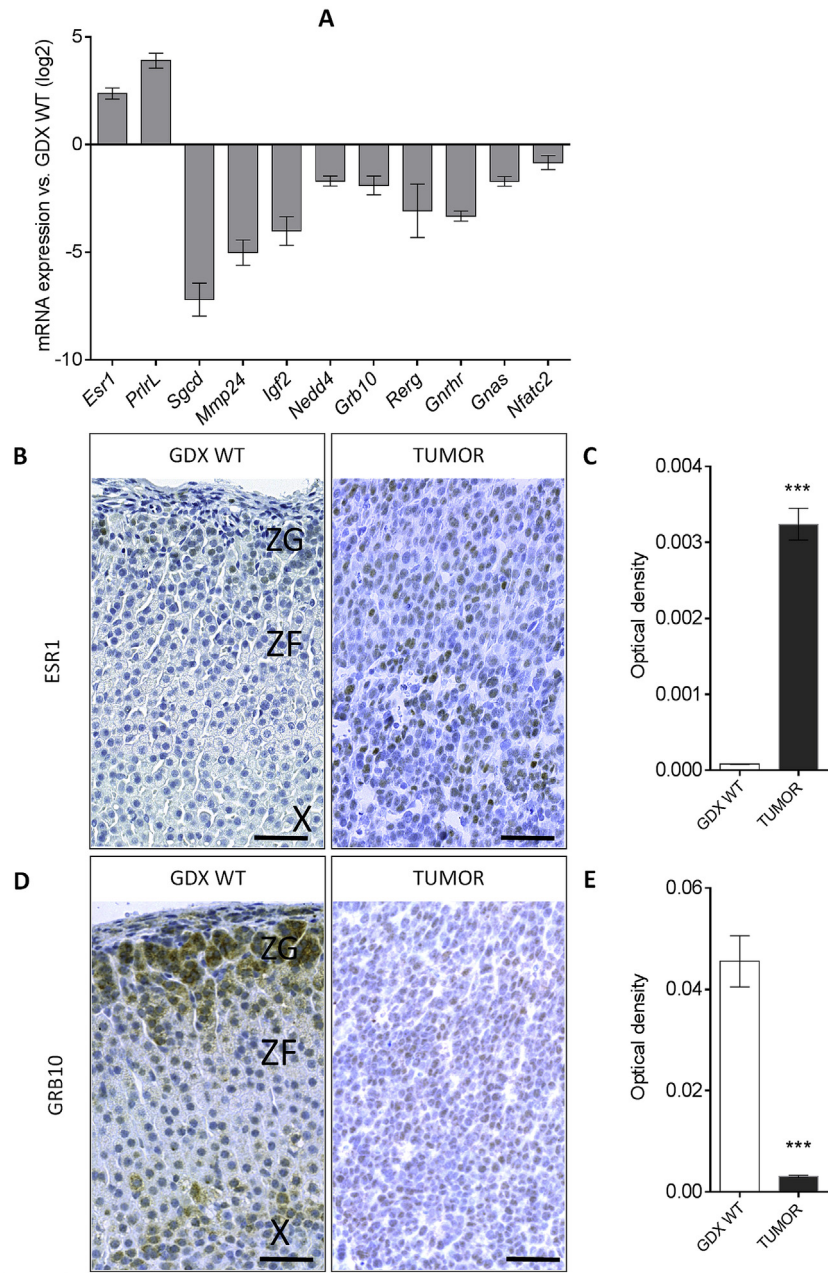


Fig. 3. Validation of the targets suggested by microarray study (part one). (A) Gene expression of significantly altered novel potential biomarker genes in GDX *inhα/Tag* males normalized by GDX WT (baseline) ($n = 8/\text{group}$). Protein localization and optical density comparison of ESR1 (B–C), GRB10 (D–E) in normal adrenal gland (GDX WT) and tumor tissue (GDX *inhα/Tag*) ($n = 4/\text{group}$). Bar = 50 μm ; ZG–zona glomerulosa, ZF– zona fasciculata, X–x-zone.

3.4. Inverted adrenal-to-gonadal steroidogenic enzyme expression profile in *inhα/Tag*

From the dysregulated pathways revealed by microarray, we further analyzed the steroid metabolic enzymes in the *inhα/Tag* adrenal tumors. GDX *inhα/Tag* mice showed significantly increased plasma levels of LH (Fig. 6A), progesterone (Fig. 6B) compared with WT and barely detectable levels of testosterone (Fig. 6C). Gene expression analysis showed significant down-regulation of adrenal (*Cyp21a1*, *Cyp11b1*, *Cyp11b2*) (Fig. 6E) but up-regulation of sex (*Srd5a1*, *Cyp19a1*) (Fig. 6E) steroidogenic enzymes. We found adrenal *Cyp17a1* was significantly increased after gonadectomy (vs. intact) in WT C57Bl/6 male mice (Supplemental Fig. S2).

4. Discussion

Chronically elevated LH levels after gonadectomy have been proposed to strain-dependently trigger and modulate *Lhcgr* expression in WT (Bernichtein et al., 2008; Bielinska et al., 2003) and transgenic mice (Kananen et al., 1996; Matzuk et al., 1994). There is also some evidence for high LH levels affecting human adrenal function (Alevizaki et al., 2006; Carlson, 2007). We did not find any difference in plasma LH levels between GDX *inhα/Tag* and WT mice. This implies the involvement of some other factors than LH action in the tumor formation. In search for such biomarkers, we ran a cDNA microarray analysis on the GDX *inhα/Tag* vs. GDX WT adrenals. A validation study confirmed two novel up-regulated

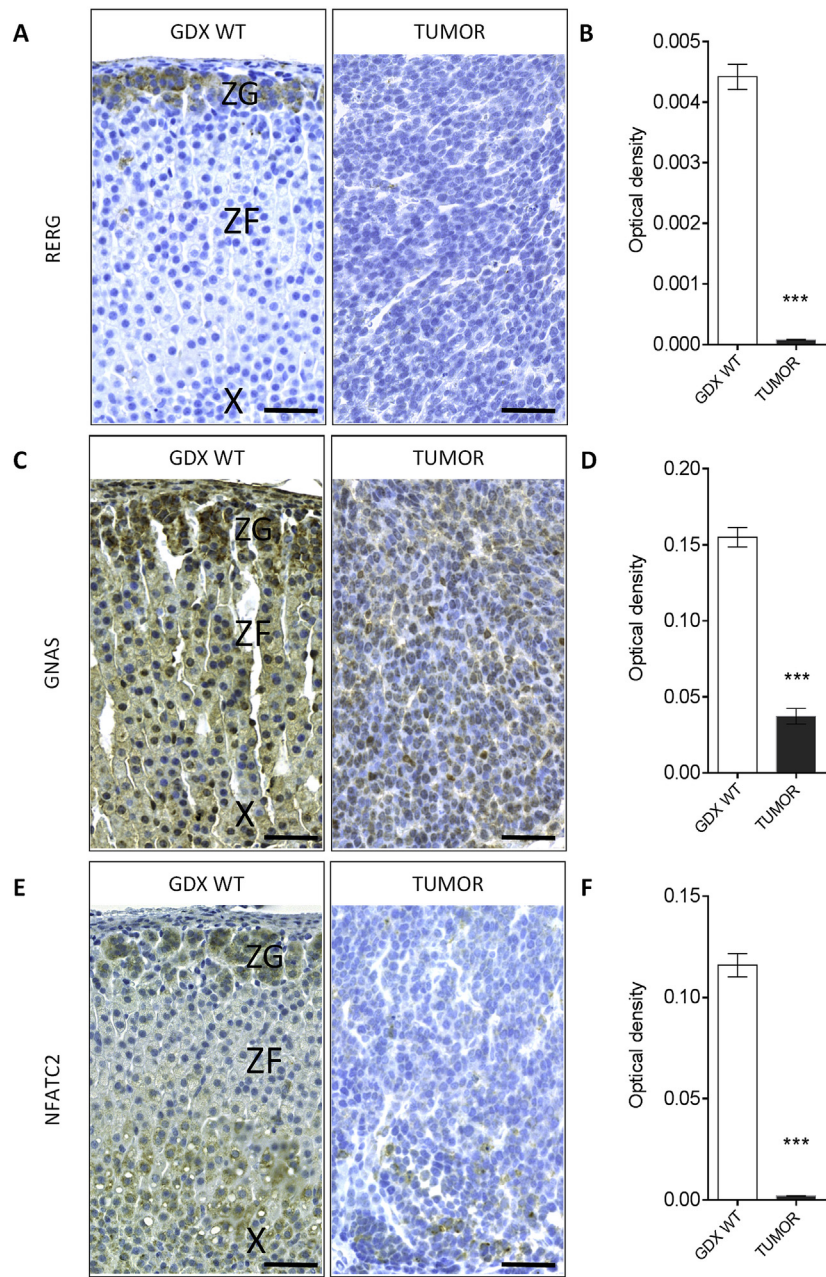


Fig. 4. Validation of the targets suggested by microarray study (part two). Protein localization and optical density measurement for RERG (A–B), GNAS (C–D), NFATC2 (E–F) in normal adrenal gland (GDX WT) vs. tumor foci (GDX inhα/Tag) of male mice (n = 4/group). Bar = 50 μm; ZG–zona glomerulosa, ZF– zona fasciculata, X–x-zone.

(*Esr1*, *Prlr-rs1*) and nine down-regulated (*Sgca*, *Mmp24*, *Igf2*, *Nedda*, *Grb10*, *Rerg*, *Gnrhr*, *Gnas*, *Nfatc2*) genes in both males and females. We did not observe any difference in protein localization between intact and GDX WT, suggesting that GDX *per se* did not affect the gene expression profile.

IHC revealed that ESR1 was expressed in normal zona glomerulosa and in a patchy manner in the tumor cells. This, as well as the up-regulated *Esr1* mRNA expression, pointed to ESR1 as a novel biomarker of inhα/Tag mouse adrenal tumors. Interestingly, *Esr1* has been shown to be expressed in B cells of gonadectomized NU/J mice (Bielinska et al., 2005) and in a subpopulation of tumor cells in domestic ferrets (Bielinska et al., 2006; Newman et al., 2004). Therefore, our data supports previous suggestions that gonadectomy-induced adrenocortical tumors may respond to

estrogen stimuli. On the other hand, normal human adrenals express both estrogen receptor alpha (ESR1) and beta (ESR2) (Barzon et al., 2008). In cases of functional ACC, ESR1 was found to be up-regulated that resulted in an increased ESR1/ESR2 ratio (Barzon et al., 2008). In our study, gene expression analysis showed *Esr1* up-regulation but no significant changes in *Esr2*. Altered ESR1/ESR2 ratio has also been described in estrogen-responsive cancers like prostate, colon, ovary and breast cancer (Bardin et al., 2004). Moreover, in the ACC derived cell line H295R, 17β-estradiol exerted proliferative effect, while antiestrogens were anti-proliferative (Montanaro et al., 2005). These observations suggest that estrogens may contribute to the progression of inhα/Tag adrenal tumors. Whether ESR1 is involved in the induction and progression of the tumors needs further investigation. It would be interesting to see

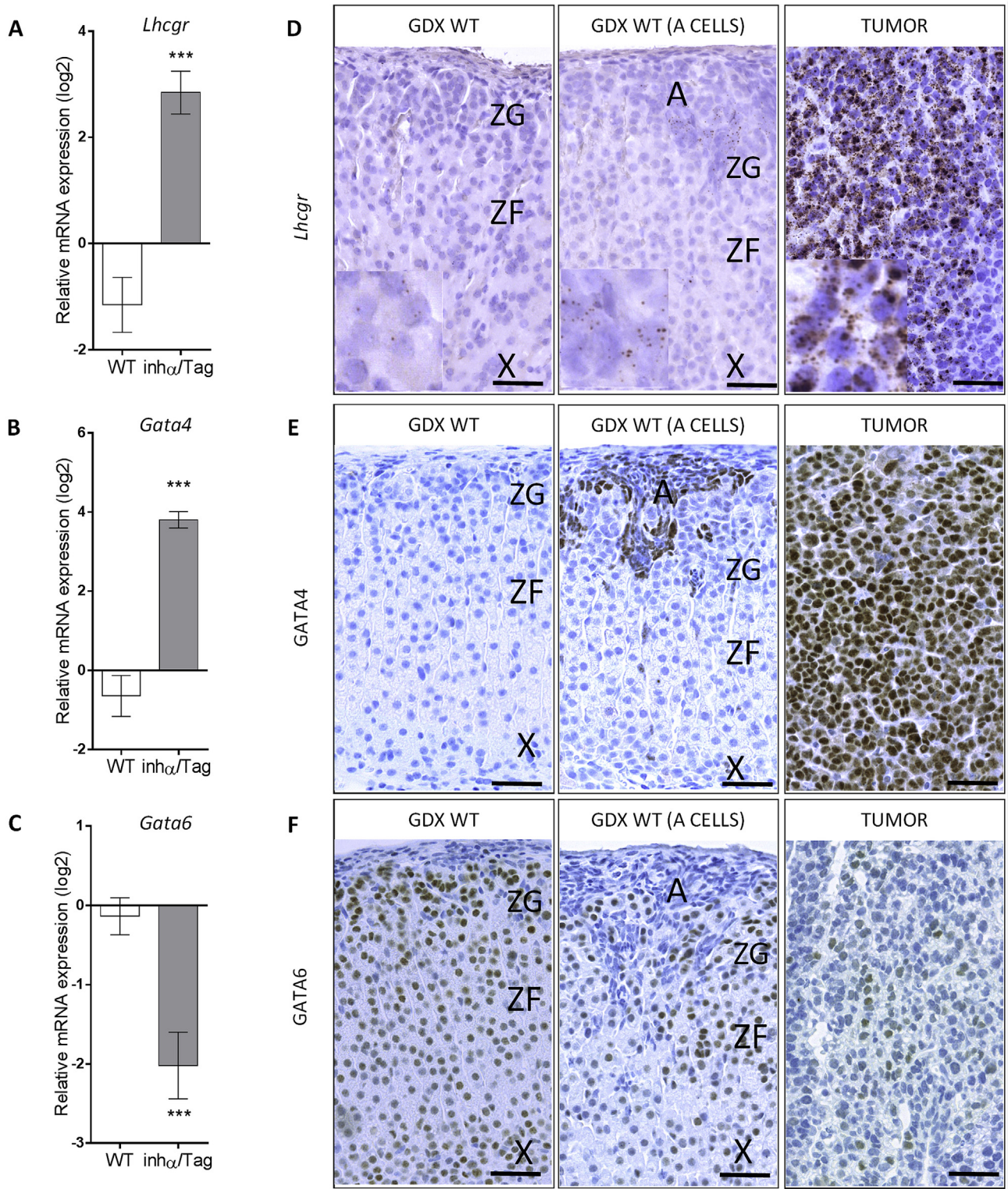


Fig. 5. Expression and localization of neoplastic cell biomarkers. Total RNA gene expression of *Lhcgr* (A), *Gata4* (B), *Gata6* (C) in the adrenals of GDx WT and GDx inh α /Tag male mice (mean \pm SEM; ***. $P \leq 0.001$; $n = 8$ /group). Localization of *Lhcgr* (D, E, F), GATA4 (G, H, I) and GATA6 (J, K, L) in the GDx WT and A cells of GDx WT, and tumor tissue of inh α /Tag mice respectively ($n = 4$ /group). Bar = 50 μ m.

the functional consequences of ESR1 knock out on tumor formation and progression, either *in vivo* by crossbreeding ESR1^{-/-} (ESR1 knock-out mice) and inh α /Tag mice, or *in vitro* by silencing the ESR1 in adrenocortical tumor cells.

PRLR-RS1 protein analysis was not doable due to the lack of antibodies for specific prolactin receptor isoforms. IHC staining results obtained with two different commercially available antibodies for PRLR gave unspecific staining in mouse tissues. Human

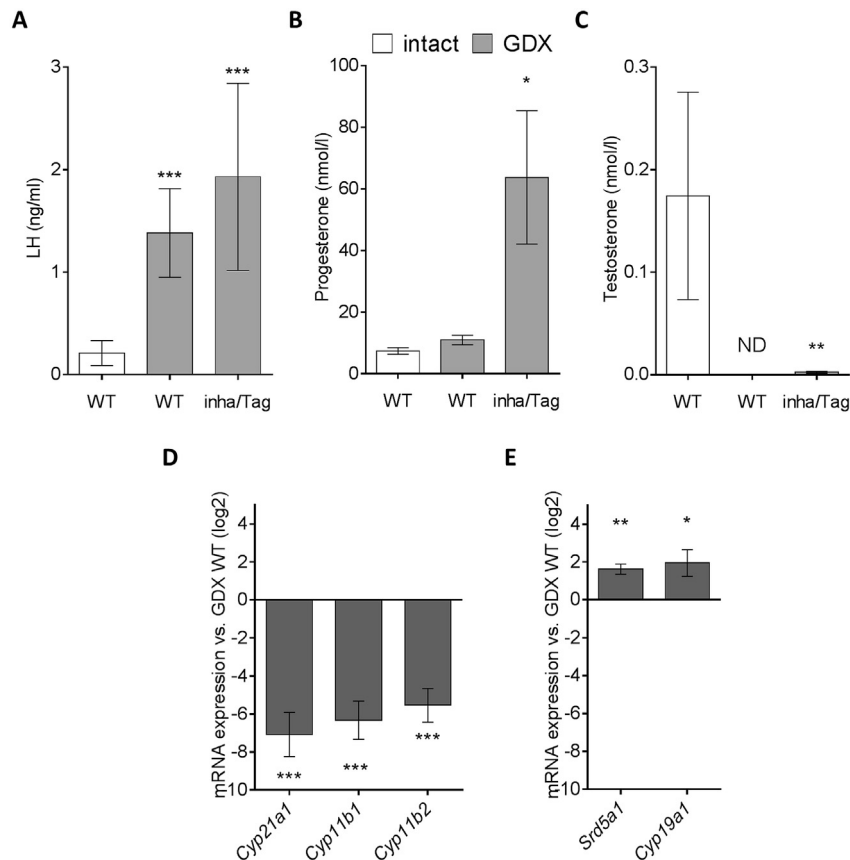


Fig. 6. Hormonal and steroidogenic enzymes profile of adrenocortical tumor bearing inhα/Tag male mice. Plasma levels of LH (A), progesterone (B), and testosterone (C) in intact WT, and GDX WT and inhα/Tag males (n = 6–9/group). Gene expression of significantly altered adrenal (D) and gonadal (E) steroidogenic enzymes in GDX inhα/Tag vs. GDX WT. (n = 8/group; mean ± SEM; *, P ≤ 0.05. **, P ≤ 0.01. ***, P ≤ 0.001.)

adrenals express functional prolactin receptors (Glasow et al., 1996). Adrenal stimulation with PRL exerts steroidogenic (Glasow et al., 1996) but not mitogenic (Glasow et al., 1998) effects. On the other hand, in bLHβ-CTP mice PRL/PRLR signaling was implicated as a regulator of LHCGR expression, which in turn lead to adrenocortical tumorigenesis (Kero et al., 2000). Therefore, it would be interesting to characterize to what extent PRL/PRLR signaling is involved in the tumor ontogeny and/or steroidogenesis in inhα/Tag mice.

The adrenal gland is one of the major sites of steroid production. Stem/progenitor cells located in the capsular, subcapsular and juxtamedullary regions divide and differentiate into cells of zona glomerulosa or zona fasciculata and x-zone (Pihlajoki et al., 2015). Moreover, zona glomerulosa and zona fasciculata cells are able to trans-differentiate into one another to replenish adrenal zones cell population rapidly (Pihlajoki et al., 2015). In this study, we localized protein expression of down-regulated target genes like GRB10, RERG, GNAS, and NFATC2. GNAS and NFATC2 are involved in GPCRs signaling (Hill et al., 2001) which seems to be more prominent in the normal adrenal vs. tumor tissue. GRB10 and RERG have not yet been characterized in the context of the adrenal cortex. Both gene products seem to inhibit cell proliferation either by inhibition of insulin and IGF1 action (Liu and Roth, 1995) or GTP hydrolysis (Finlin et al., 2001), respectively. Their abundant expression in subcapsular and zona glomerulosa regions suggests their involvement in the maintenance of adrenocortical zonation and perhaps in the prevention of overgrowth of that region. Although, putative tumor suppressor role of these downregulated genes could not be ruled out.

The dysregulated GPCR signaling pathways indicated by GO analysis prompted us to revisit the *Lhcgr* expression. LHCGR has been previously described as a possible trigger for adrenocortical tumorigenesis in inhα/Tag model (Kananen et al., 1996; Mikola et al., 2003). There is a lack of commercially available good-quality antibodies against LHCGR. Thus, in this study, we determined *Lhcgr* localization by RNAscope *in situ* hybridization (Wang et al., 2012). *Lhcgr* transcripts could be detected in normal adrenocortical cells and in A cells of 6-mo intact and GDX WT C57BL/6N animals. Moreover, we confirmed the robust *Lhcgr* expression in the foci of inhα/Tag adrenocortical tumor cells (Rahman et al., 2001). The expression of *Lhcgr* in A cells has been reported only in GDX DBA/2J adrenals when accompanied by B cells in gonadotropin-induced adrenocortical tumors (Bielinska et al., 2003), but not in the non-susceptible C57bl/6N mouse strain. The role and expression of *Lhcgr* in normal mouse adrenal gland remains questionable since it does not produce androgens (Pihlajoki et al., 2015), albeit a detectable mRNA level of *Lhcgr* expression has been shown by PCR-based methods (Chruscziel et al., 2013; Looyenga and Hammer, 2006). Interestingly, the pattern of *Lhcgr* localization in normal cortex is identical in intact and GDX WT animals (Fig. S1), although total RNA analysis in many studies showed up-regulated levels of *Lhcgr* in aging mice after gonadectomy (Chruscziel et al., 2013; Kero et al., 2000; Looyenga and Hammer, 2006). Recent lineage tracing studies showed an increase of A cells population after gonadectomy (Bandiera et al., 2013). This may suggest that the up-regulated *Lhcgr* expression after gonadectomy (chronically elevated LH levels) could be associated with the increase in A cell population, whereas *Lhcgr* transcripts in normal intact adrenal remain unchanged. One

of the current theories of steroidogenic lipid-laden B cells origin suggests that A cells differentiate into B cells upon exposure to high LH concentration (Bielinska et al., 2003). This suggests that the *Lhcgr* expression in A cells could be crucial trigger for B cell formation. Therefore, it is possible that despite their gonadotropin independent induction, A cells could respond to LH action later on.

The reciprocal expression of GATA4 and GATA6 in the adrenocortical tumors of *inhα/Tag* and *Inha*^{−/−} mice has been reported earlier (Looyenga and Hammer, 2006; Vuorenoja et al., 2007). In our study GATA4 expression appeared to be restricted to neoplastic A cells (GDX WT and *inhα/Tag*) and tumor tissue (*inhα/Tag*), and no staining was seen in normal adrenal tissue. Since GATA4 was also expressed in B cells of GDX DBA/2J (Chrusciel et al., 2013; Krachulec et al., 2012) and in adrenal carcinoma cells of *Inha*^{−/−} mice (Looyenga and Hammer, 2006), we could confirm that GATA4 could be used as a neoplastic cell marker in mouse adrenal tumor models. GATA6 expression was found mainly in normal adrenocortical cells, and in some tumor cells, but it was never found in A cells. Recently it was shown that GATA6 is crucial for normal adrenal development and when absent, adrenocortical progenitors tend to differentiate into neoplastic A cells (Pihlajoki et al., 2013). In turn, GATA4 triggers neoplastic B-cell formation (Chrusciel et al., 2013; Krachulec et al., 2012) and is most likely responsible for their gonadal-like gene expression profile (Chrusciel et al., 2013; Krachulec et al., 2012; Looyenga and Hammer, 2006). However, in transgenic mice expressing GATA4 under the 21-hydroxylase promoter (21-OH-GATA-4), GATA4 expression was not sufficient to cause B-cell formation without a gonadectomy-induced gonadotropin boost (Chrusciel et al., 2013). Most likely in all gonadectomy-induced adrenocortical tumorigenesis models, gonadotropins act upstream of the tumorigenesis, but the mechanism of their involvement in this process remains unknown. Until now, only in the *Inha*^{−/−} mouse model this mechanism has been proposed and FSH was identified to work as an upstream inductor to switch on the GATA6 to GATA4 transition (Looyenga and Hammer, 2006). In *Inha*^{−/−} mice, similarly to our studies, attenuation of GATA6 and induction of GATA4 expression in neoplastic tissue was observed (Looyenga and Hammer, 2006). It would be interesting to see if the silencing of the LH/LHCGR signaling in *inhα/Tag* or in DBA/2J or *Inha*^{−/−} mice would block the adrenocortical tumorigenesis.

Enrichment analysis of the microarray data indicated dysregulation of steroid biosynthesis and metabolism. Validation data showed that tumor cells in *inhα/Tag* mice have decreased expression of genes of adrenal steroidogenic pathway and rather express sex steroidogenic enzymes. Interestingly, in contrast to significantly increased *Cyp19a1* and *Srd5a1*, expression of *Cyp17a1* was not altered in our experimental model (GDX *inhα/Tag* vs. GDX WT). A recent study showed a significant increase of adrenal CYP17A1 expression after gonadectomy (vs. intact) in a C57Bl/6 background (Bandiera et al., 2013). We also found significantly increased adrenal *Cyp17a1* after gonadectomy in WT C57Bl/6 mice. Our results suggest that in C57Bl/6 background, *Cyp17a1* expression could be upregulated in WT C57Bl/6 adrenal as a consequence of gonadectomy, but not due to adrenocortical tumor onset as it was observed in gonadectomized DBA/2J mice (Bielinska et al., 2003; Krachulec et al., 2012). Production of estrogen by tumorous adrenals of GDX DBA/2J mice (Bielinska et al., 2003, 2005) and low levels of testosterone found in GDX *inhα/Tag* males indicates that the sex steroid production is a common feature in models susceptible to gonadotropin dependent tumorigenesis (Bielinska et al., 2003, 2005; Krachulec et al., 2012).

The counterpart to gonadectomy-induced adrenocortical tumors are the adrenal rest tumors in gonads, where high adrenocorticotropin (ACTH) levels induce adrenal cell-like tumors in gonads (Claahsen-Van der Grinten et al., 2009). The current

theories claim that they originate from either adrenal progenitors 'trapped' in gonads or somatic testicular cells expressing receptors for ACTH (MC2R) (Claahsen-Van der Grinten et al., 2009). Therefore, elevated ACTH levels (i.e. as a result of congenital adrenal hyperplasia) stimulate expression of adrenal markers and drive formation of benign testicular adrenal rest tumors (Claahsen-Van der Grinten et al., 2009).

In conclusion, we found ESR1 as a potential biomarker of adrenocortical tumorigenesis in *inhα/Tag* mice. In functional ACC, ESR1 has been already found to be up-regulated, thus the clinical relevance is already there, although this has not been studied further (Barzon et al., 2008). We also identified novel genes, *Grb10*, *Rerg*, *Gnas*, and *Nfatc2*, which are most likely involved in normal adrenal remodeling and/or as tumor suppressor genes. We also found low levels of *Lhcgr* transcripts in normal adrenocortical cells and in neoplastic A cells of intact and GDX C57Bl/6N WT mice. Adrenocortical tumors bearing *inhα/Tag* mice presented with an inverted adrenal-to-gonadal profile of steroidogenic enzymes expression. It would be of great importance to check the genes with suppressed expression in human ACT, as this mouse model tumorigenesis resembles human tumors. Thus these genes may have human relevance and could be further considered as potential diagnostic and therapeutic tools in human tumors with similar molecular pathogenesis.

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Author contributions

MD, MC, NR designed the study concept; MD, MC, KB, SV performed the experiments; all the authors analyzed and interpreted the results; MD, MC, IH, JT and NR drafted the manuscript and all the authors have approved the final manuscript.

Declaration of interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2017.01.036>.

References

- Alevizaki, M., Saltiki, K., Mantzou, E., Anastasiou, E., Huhtaniemi, I., 2006. The adrenal gland may be a target of LH action in postmenopausal women. *Eur. J. Endocrinol.* 154, 875–881.
- Bandiera, R., Vidal, V.P., Motamedi, F.J., Clarkson, M., Sahut-Barnola, I., Von Gise, A., Pu, W.T., Hohenstein, P., Martinez, A., Schedl, A., 2013. WT1 maintains adrenal-gonadal primordium identity and marks a population of AGP-like progenitors within the adrenal gland. *Dev. Cell* 27, 5–18.
- Bardin, A., Bouille, N., Lazennec, G., Vignon, F., Pujol, P., 2004. Loss of ERβ expression as a common step in estrogen-dependent tumor progression. *Endocrine-related*

- Cancer 11, 537–551.
- Barzon, L., Masi, G., Pacenti, M., Trevisan, M., Fallo, F., Remo, A., Martignoni, G., Montanaro, D., Pezzi, V., Pali, G., 2008. Expression of aromatase and estrogen receptors in human adrenocortical tumors. *Virchows Arch.* 452, 181–191.
- Bernichtein, S., Petretto, E., Jamieson, S., Goel, A., Aitman, T.J., Mangion, J.M., Huhtaniemi, I.T., 2008. Adrenal gland tumorigenesis after gonadectomy in mice is a complex genetic trait driven by epistatic loci. *Endocrinology* 149, 651–661.
- Bielinska, M., Genova, E., Boime, I., Parviainen, H., Kiiveri, S., Leppaluoto, J., Rahman, N., Heikinheimo, M., Wilson, D.B., 2005. Gonadotropin-induced adrenocortical neoplasia in NU/J nude mice. *Endocrinology* 146, 3975–3984.
- Bielinska, M., Kiiveri, S., Parviainen, H., Mannisto, S., Heikinheimo, M., Wilson, D.B., 2006. Gonadectomy-induced adrenocortical neoplasia in the domestic ferret (*Mustela putorius furo*) and laboratory mouse. *Vet. Pathol.* 43, 97–117.
- Bielinska, M., Parviainen, H., Porter-Tinge, S.B., Kiiveri, S., Genova, E., Rahman, N., Huhtaniemi, I.T., Muglia, L.J., Heikinheimo, M., Wilson, D.B., 2003. Mouse strain susceptibility to gonadectomy-induced adrenocortical tumor formation correlates with the expression of GATA-4 and luteinizing hormone receptor. *Endocrinology* 144, 4123–4133.
- Carlson, H.E., 2007. Human adrenal cortex hyperfunction due to LH/hCG. *Mol. Cell Endocrinol.* 269, 46–50.
- Chrusciel, M., Vuorenoja, S., Mohanty, B., Rivero-Muller, A., Li, X.D., Toppari, J., Huhtaniemi, I., Rahman, N.A., 2013. Transgenic GATA-4 expression induces adrenocortical tumorigenesis in C57Bl/6 mice. *J. Cell Sci.* 126, 1845–1857.
- Claahsen-Van der Grinten, H., Hermus, A., Otten, B., 2009. 2009 Testicular adrenal rest tumours in congenital adrenal hyperplasia. *Int. J. Pediatr. Endocrinol.* 1.
- Eden, E., Navon, R., Steinfeld, I., Lipson, D., Yakhini, Z., 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinforma.* 10, 48.
- Finlin, B.S., Gau, C.-L., Murphy, G.A., Shao, H., Kimel, T., Seitz, R.S., Chiu, Y.-F., Botstein, D., Brown, P.O., Der, C.J., 2001. RERG is a novel ras-related, estrogen-regulated and growth-inhibitory gene in breast cancer. *J. Biol. Chem.* 276, 42259–42267.
- Glasow, A., Breidert, M., Haidan, A., Anderegg, U., Kelly, P.A., Bornstein, S.R., 1996. Functional aspects of the effect of prolactin (PRL) on adrenal steroidogenesis and distribution of the PRL receptor in the human adrenal gland. *J. Clin. Endocrinol. Metabolism* 81, 3103–3111.
- Glasow, A., Haidan, A., Gillespie, J., Kelly, P., Chrousos, G., Bornstein, S., 1998. Differential expression of prolactin receptor (PRLR) in normal and tumorous adrenal tissues: separation of cellular endocrine compartments by laser capture microdissection (LCM). *Endocr. Res.* 24, 857–862.
- Haavisto, A.M., Pettersson, K., Bergendahl, M., Perheentupa, A., Roser, J.F., Huhtaniemi, I., 1993. A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology* 132, 1687–1691.
- Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8, 1901–1914.
- Hill, S.J., Baker, J.G., Rees, S., 2001. Reporter-gene systems for the study of G-protein-coupled receptors. *Curr. Opin. Pharmacol.* 1, 526–532.
- Hofmann, F.G., Dickie, M.M., Christy, N.P., 1960. Studies of gonadectomized mice bearing adrenal cortical tumours. *Acta Endocrinol. (Copenh)* 34, 84–96.
- Hudson, A.L., Colvin, E.K., 2016. Transgenic mouse models of SV40-induced cancer. *ILAR J.* 57, 44–54.
- Kananen, K., Markkula, M., Mikola, M., Rainio, E.M., McNeilly, A., Huhtaniemi, I., 1996. Gonadectomy permits adrenocortical tumorigenesis in mice transgenic for the mouse inhibin alpha-subunit promoter/simian virus 40 T-antigen fusion gene: evidence for negative autoregulation of the inhibin alpha-subunit gene. *Mol. Endocrinol.* 10, 1667–1677.
- Kero, J., Poutanen, M., Zhang, F.P., Rahman, N., McNicol, A.M., Nilson, J.H., Keri, R.A., Huhtaniemi, I.T., 2000. Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. *J. Clin. Invest.* 105, 633–641.
- Kiiveri, S., Siltanen, S., Rahman, N., Bielinska, M., Lehto, V.P., Huhtaniemi, I.T., Muglia, L.J., Wilson, D.B., Heikinheimo, M., 1999. Reciprocal changes in the expression of transcription factors GATA-4 and GATA-6 accompany adrenocortical tumorigenesis in mice and humans. *Mol. Med.* 5, 490–501.
- Krachulec, J., Vetter, M., Schrade, A., Lobs, A.K., Bielinska, M., Cochran, R., Kyronlahti, A., Pihlajoki, M., Parviainen, H., Jay, P.Y., et al., 2012. GATA4 is a critical regulator of gonadectomy-induced adrenocortical tumorigenesis in mice. *Endocrinology* 153, 2599–2611.
- Laufer, E., Kesper, D., Vortkamp, A., King, P., 2012. Sonic hedgehog signaling during adrenal development. *Mol. Cell Endocrinol.* 351, 19–27.
- Lerario, A.M., Moraitis, A., Hammer, G.D., 2014. Genetics and epigenetics of adrenocortical tumors. *Mol. Cell Endocrinol.* 386, 67–84.
- Levine, A.J., Momand, J., Finlay, C.A., 1991. The p53 tumour suppressor gene. *Nature* 351, 453–456.
- Liu, F., Roth, R.A., 1995. Grb-IR: a SH2-domain-containing protein that binds to the insulin receptor and inhibits its function. *Proc. Natl. Acad. Sci.* 92, 10287–10291.
- Looyenga, B.D., Hammer, G.D., 2006. Origin and identity of adrenocortical tumors in inhibin knockout mice: implications for cellular plasticity in the adrenal cortex. *Mol. Endocrinol.* 20, 2848–2863.
- Matzuk, M.M., Finegold, M.J., Mather, J.P., Krummen, L., Lu, H., Bradley, A., 1994. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. *Proc. Natl. Acad. Sci. U S A.* 91, 8817–8821.
- Mikola, M., Kero, J., Nilson, J.H., Keri, R.A., Poutanen, M., Huhtaniemi, I., 2003. High levels of luteinizing hormone analog stimulate gonadal and adrenal tumorigenesis in mice transgenic for the mouse inhibin- α -subunit promoter/Simian virus 40 T-antigen fusion gene. *Oncogene* 22, 3269–3278.
- Montanaro, D., Maggiolini, M., Recchia, A., Sirianni, R., Aquila, S., Barzon, L., Fallo, F., Ando, S., Pezzi, V., 2005. Antiestrogens upregulate estrogen receptor β expression and inhibit adrenocortical H295R cell proliferation. *J. Mol. Endocrinol.* 35, 245–256.
- Moreno, S., Guillermo, M., Decouls, M., Dewailly, D., Bresson, R., Proye, C., 2006. Feminizing adreno-cortical carcinomas in male adults. A dire prognosis: three cases in a series of 801 adrenalectomies and review of the literature. In: *Annales D'endocrinologie*. Elsevier, pp. 32–38.
- Newman, S., Bergman, P., Williams, B., Scase, T., Craft, D., 2004. Characterization of spindle cell component of ferret (*Mustela putorius furo*) adrenal cortical neoplasms—correlation to clinical parameters and prognosis. *Veterinary Comp. Oncol.* 2, 113–124.
- Pihlajoki, M., Dörner, J., Cochran, R.S., Heikinheimo, M., Wilson, D.B., 2015. Adrenocortical zonation, renewal, and remodeling. *Front. Endocrinol.* 6, 27.
- Pihlajoki, M., Gretzinger, E., Cochran, R., Kyronlahti, A., Schrade, A., Hiller, T., Sullivan, L., Shoykhet, M., Schoeller, E.L., Brooks, M.D., et al., 2013. Conditional mutagenesis of Gata6 in SF1-positive cells causes gonadal-like differentiation in the adrenal cortex of mice. *Endocrinology* 154, 1754–1767.
- Rahman, N.A., Kiiveri, S., Rivero-Muller, A., Levallet, J., Vierre, S., Kero, J., Wilson, D.B., Heikinheimo, M., Huhtaniemi, I., 2004. Adrenocortical tumorigenesis in transgenic mice expressing the inhibin alpha-subunit promoter/simian virus 40 T-antigen transgene: relationship between ectopic expression of luteinizing hormone receptor and transcription factor GATA-4. *Mol. Endocrinol.* 18, 2553–2569.
- Rahman, N.A., Kiiveri, S., Siltanen, S., Levallet, J., Kero, J., Lensu, T., Wilson, D.B., Heikinheimo, M.T., Huhtaniemi, I.T., 2001. Adrenocortical tumorigenesis in transgenic mice: the role of luteinizing hormone receptor and transcription factors GATA-4 and GATA-6. *Reprod. Biol.* 1, 5–9.
- Rilianawati, Kero, J., Paukku, T., Huhtaniemi, I., 2000. Long-term testosterone treatment prevents gonadal and adrenal tumorigenesis of mice transgenic for the mouse inhibin-alpha subunit promoter/simian virus 40 T-antigen fusion gene. *J. Endocrinol.* 166, 77–85.
- Rilianawati, Paukku T., Kero, J., Zhang, F.P., Rahman, N., Kananen, K., Huhtaniemi, I., 1998. Direct luteinizing hormone action triggers adrenocortical tumorigenesis in castrated mice transgenic for the murine inhibin alpha-subunit promoter/simian virus 40 T-antigen fusion gene. *Mol. Endocrinol.* 12, 801–809.
- Risma, K.A., Clay, C.M., Nett, T.M., Wagner, T., Yun, J., Nilson, J.H., 1995. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc. Natl. Acad. Sci. U S A.* 92, 1322–1326.
- Rosner, J.M., Charreau, E., Houssay, A.B., Epper, C., 1966. Biosynthesis of sexual steroids by hyperplastic adrenal glands of castrated female C3H/Ep mice. *Endocrinology* 79, 681–686.
- Saner-Amigh, K., Mayhew, B.A., Mantero, F., Schiavi, F., White, P.C., Rao, C.V., Rainey, W.E., 2006. Elevated expression of luteinizing hormone receptor in aldosterone-producing adenomas. *J. Clin. Endocrinol. Metabolism* 91, 1136–1142.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., 2012. Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682.
- Stojadinovic, A., Brennan, M.F., Hoos, A., Omeroglu, A., Leung, D.H., Dudas, M.E., Nissán, A., Cordon-Cardo, C., Ghossein, R.A., 2003. Adrenocortical adenoma and carcinoma: histopathological and molecular comparative analysis. *Mod. Pathol.* 16, 742–751.
- Vuorenoja, S., Rivero-Muller, A., Kiiveri, S., Bielinska, M., Heikinheimo, M., Wilson, D.B., Huhtaniemi, I.T., Rahman, N.A., 2007. Adrenocortical tumorigenesis, luteinizing hormone receptor and transcription factors GATA-4 and GATA-6. *Mol. Cell Endocrinol.* 269, 38–45.
- Wang, F., Flanagan, J., Su, N., Wang, L.C., Bui, S., Nielson, A., Wu, X., Vo, H.T., Ma, X.J., Luo, Y., 2012. RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diagn.* 14, 22–29.